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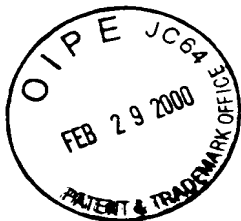
In re application of:

Podhajcer *et al.*

Appl. No. 09/340,595

Filed: June 28, 1999

For: **Compositions and Methods for
Tumour Therapy**



Art Unit: 1635

Examiner: To Be Assigned

Atty. Docket: 1581.0300002/RWE/KKV

Claim For Priority Under 35 U.S.C. § 119(a)-(d) In Utility Application

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Priority under 35 U.S.C. § 119(a)-(d) is hereby claimed to the following priority document, filed in a foreign country within twelve (12) months prior to the filing of the above-referenced United States utility patent application:

Country	Priority Document Appl. No.	Filing Date
Great Britain	9626989.9	December 27, 1996

A certified copy of each listed priority document is submitted herewith. Prompt acknowledgment of this claim and submission is respectfully requested.

Respectfully submitted,

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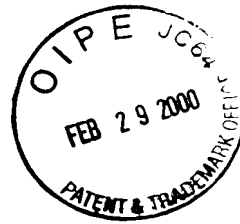




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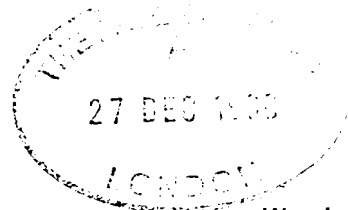
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— NOT APPLICABLE —

Products and processes for gene transfer and regulation

Field of the Invention

This invention relates to vectors and to the regulation of cellular activity, especially for example by methods of using the vectors to transduce cells, e.g. malignant cells, and to induce expression of foreign genetic material in such cells to regulate cellular activity, particularly cellular activity that is dependent on the action of osteonectin. The invention also relates to pharmaceutical compositions based on such vectors, to production of cells transduced or transfected with such vectors and their expression products, to pharmaceutical preparations based on such cells, to their use for administration to humans and non-human animals, to their use in order to express foreign genetic material in vivo and in vitro, and to the use of materials as described herein for the manufacture of preparations for treatment and other applications as mentioned herein. Methods and products according to the invention can be used for example in cancer immunotherapy.

Background of the invention and Prior Art

Osteonectin is a known protein, otherwise designated as SPARC (secreted protein, acidic and cysteine-rich), or as BM40 and 43Kd. It is a secreted glycoprotein widely distributed in human and non-human animal tissues. It is associated with cell populations exhibiting high rates of turnover and remodelling [5,6]. The protein is secreted by different normal cells as a unique glycoprotein band of 40-44 kDa [5]. It has been shown that in addition to a reactive protein doublet of 40-45 kDa, osteonectin can appear in fresh melanoma samples and cell lines as a cleaved protein doublet of 34-35 kDa. Genes that encode osteonectin in several species, including the human, are also known. The gene encoding osteonectin is known to be highly conserved across a wide range of living organisms.

A diverse range of functions and effects has been associated with osteonectin. It has been found to interact with extracellular matrix components, growth factors, cytokines, and to regulate matrix metalloproteinase expression.

The literature of oncology includes numerous mentions of osteonectin and its synonyms. In particular, reports from different laboratories indicate that osteonectin overexpression was associated with neoplastic progression of different malignant tumours [8-12], including human melanoma. Porter et al [8] previously reported increased SPARC expression in ovary cancer cells, but on the other hand Mock et al have reported that SPARC expression is down-regulated in ovary cancer cells compared to normal cells [27], and that moreover the ~~stable overexpression~~ overexpression in ovary cancer cell lines of SPARC, secreted as a unique species of 43 kDa, reduced their growth capacity [27]. Molecular analysis of SPARC role in cell

growth has to take into account studies (including data of the present inventors) showing no effect on melanoma cell growth following SPARC down-regulation.

5 Osteonectin expression has been reported to be correlated with lung colonization by tumours. Increased expression has been reported in human breast cancer. Neoplastic progression of colorectal cancer has been reported as associated with overexpression of certain proteins including osteonectin.

10 Osteonectin has been reported as having effects on tumour cell adhesion and invasion. One view concerning cell-matrix interaction in tumour progression indicates that inhibition of tumour cell adhesion can result in less aggressive invasive behaviour. Adhesive proteins like fibronectin, vitronectin and laminin can regulate metastatic activity, invasive capacity and collagenase IV production of human and murine melanoma cells [13-16]. Osteonectin by contrast is considered to be a counteradhesive protein involved in matrix deposition and
15 assembly due to its interaction with collagens types I-V [5,6,17], thrombospondin [5,18] and plasminogen [18] and the regulation of the expression levels of laminin [19], fibronectin [18,19], matrix metalloproteinases [7] and plasminogen activator inhibitor-1 [18].

20 The present inventors consider it possible that osteonectin cleavage by melanoma cells and the release of specific protein domains can greatly alter the functional role of the parent protein. Peptides corresponding to different SPARC domains have shown specific activities on cell proliferation, shape regulation and binding to collagen which were sometimes masked in the whole protein [5].

25 Domains I and IV of SPARC, which can be specifically cleaved and released by different serine proteases [20], have been found responsible for SPARC binding to collagen IV and for disruption of focal cell adhesions [21,22].

30 Native SPARC and a specific peptide corresponding to domain II which exhibit sequence similarity with EGF-like domains have been shown to inhibit endothelial cell proliferation [23]. However, a second peptide corresponding to another region of domain II containing the Cu^{2+} binding sequence KKGHK which promotes cell growth and angiogenesis [24] stimulated endothelial cells and fibroblast proliferation [23].

35 The present inventors consider that SPARC fragments produced by melanoma cells could act by either blocking the normal interaction of native SPARC with a cell surface receptor, or binding to a receptor in a different mode than native SPARC, causing changes in signal transduction, leading eventually to altered expression of gelatinase A. These fragments may

also interfere with SPARC interactions with other matrix components, altering matrix assembly, the distribution of cytoskeletal elements, and hence tumour cell adhesiveness.

5 Sage and colleagues reported that SPARC was able to inhibit DNA synthesis of endothelial cells, through a mechanism which did not involve changes in cell morphology [28]. Their evidence that peptides corresponding to domain II of SPARC can stimulate cell proliferation [23], suggests that the extracellular cleavage may have important consequences on the ability of SPARC to modulate cell growth. On the other hand, SPARC obtained from bovine bone, which efficiently modulated laminin and fibronectin secretion, was reported to have negligible effects on cell growth of different human malignant cell lines [19]. Stable over- or under-expression of SPARC in F9 cells, which correlated with altered morphologies in transfected cells, was reported not to affect cell growth [29]. In general, SPARC interaction with and regulation of the expression levels of matrix components seems to occur in the absence of any significant effect on cell growth [5,6]. Taken together, data from the literature and data of the present findings suggest that SPARC inhibition of cell growth can be related to specific cell types, probably independent of its modulation of cell matrix interaction.

20 Tumour cell rejection has been associated with a localized and massive inflammatory infiltrate of segmented neutrophils suggesting an increased availability of neutrophil trigger factors like IL-8 or the autocrine melanoma growth stimulating activity (MGSA/GRO) [30]. Matrix components like laminin were shown to modulate the availability of cytokines and chemokines [31] indicating that changes in matrix composition or in the interactions between tumour cells and matrix components may alter the immunological signals transduced to the immune system.

25 The concept of antisense strategies involving oligodeoxynucleotides and plasmid-derived RNA has previously been proposed as an attractive mode of anticancer gene therapy [32].

30 In certain cases the constitutive expression of antisense RNA coding for particular oncogenes and growth factors has been reported to be effective in the induction of reduced tumorigenicity or of in vitro malignant characteristics [33-36]. But, with few exceptions, complete suppression of tumour formation has not been achieved [32]. Accordingly, there remains a need for further materials and methods that can be used in the characterization and therapy of malignant disease and the modulation of tumour cell activity.

35 The Present Invention:

The present inventors have found that cellular downregulation of osteonectin in tumour cells can be associated with loss of tumorigenic effect, and that the effect can be transmitted

from downregulated tumour cells to neighbouring untreated tumour cells. Downregulation of osteonectin can in particular cause decrease in in-vitro adhesive and invasive capacity of tumour cells, and the present inventors consider that this is usefully connected with the loss of tumorigenic effect.

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For example, it has been found that SPARC (osteonectin) inhibition in melanoma cells can suppress their tumorigenic capacity, providing the ability to use SPARC gene targeting for antisense therapy of human melanoma.

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Evidence has been found, in particular, that SPARC (osteonectin) plays a key role in the tumorigenic capacity of human melanoma. It has been found for example that downregulation of SPARC completely prevented tumour formation in nude mice. This effect was accompanied by a massive immune response in vivo and a strong decrease in the in vitro adhesive and invasive capacity of tumour cells. The present inventors have observed that with SPARC antisense-transfected cells no initial tumour formation was visible and tumour cell necrosis was observed very rapidly. This indicates that SPARC antisense-transfected cells were impaired in their ability to establish early tumour growth.

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Accordingly, the present invention provides materials including vectors for downregulating osteonectin and processes for their use in transforming human and animal cells, particularly malignant cells, to downregulate osteonectin. Included in the invention is the provision of materials and methods to produce transfer of genetic material, with applicability to a useful range of target cell types to downregulate osteonectin. Materials provided according to the invention include materials that can be used as vaccines for tumour immunotherapy.

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The invention provides inter alia: antisense polynucleotide encoding RNA that is complementary to mRNA encoding osteonectin. According to the invention the antisense polynucleotide can be used for cellular downregulation of osteonectin, and can also be used for cellular downregulation of osteonectin and transmission of loss of tumorigenic effect from downregulated cells to neighbouring untreated cells.

35

The invention further provides vectors for transfer of heterologous genetic material into a cell, comprising the antisense polynucleotide: e.g. viral and non-viral vectors. The vectors can be used according to the invention for transforming human and animal cells, e.g. malignant cells, to downregulate osteonectin.

Also provided by the invention are vaccine compositions for tumour immunotherapy, comprising vectors as mentioned herein. The vaccines can also contain further components

such as carriers, excipients, and adjuvants.

Cells treated ex-vivo with the antisense polynucleotide can be used according to the invention to downregulate the effects of osteonectin protein.

5

The invention also provides cells comprising the downregulator arranged so as to express constitutively the downregulatory antisense nucleic acid.

10

According to an aspect of the invention there is provided a material to regulate cellular activity that is dependent on the action of osteonectin, for example to downregulate the effects of osteonectin protein in cells. The material can for example comprise a vector containing nucleic acid that encodes a downregulator for osteonectin. Such a downregulator can for example comprise nucleic acid encoding antisense RNA against osteonectin mRNA. In useful examples the downregulator can be arranged, e.g. with a

15

CMV IE promoter or other suitable promoter, so that the downregulatory antisense nucleic acid can be constitutively expressed as RNA in the treated cell.

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According to a further aspect of the invention there are provided cells in which the effects of osteonectin protein have been downregulated by treatment according to the invention, e.g. so that they carry and transcribe DNA encoding antisense RNA complementary to osteonectin mRNA.

25

Vectors according to the invention can be for example plasmids or viral vectors, e.g. recombinant viral vectors based on retroviruses, adenoviruses, herpesviruses, and/or others, encoding a nucleic acid sequence that is downregulatory for osteonectin. Such a downregulatory nucleic acid sequence can for example comprise antisense nucleic acid against osteonectin-encoding nucleic acid, e.g. DNA encoding antisense RNA against osteonectin mRNA.

30

Also provided by the invention is naked DNA, e.g. DNA oligonucleotides encoding antisense for osteonectin, and corresponding DNA enveloped or encapsulated in non-viral delivery compositions, e.g. in liposomes. Alternative embodiments encode antisense for osteonectin in plasmid-derived RNA.

35

Such vectors and other materials provided according to the invention can be used either in-vivo or ex-vivo.

Examples of vectors as provided herein can be used for direct injection of solid tumours.

Examples of vectors as provided herein can be used for ex-vivo transfection of cells (e.g. tumour cells). Cells transfected ex-vivo can be used for infusion into a subject to be treated, optionally after culture and/or cell killing, e.g. by irradiation. The transfected cells can also be transfected with a gene that renders them selectively sensitive to killing by a pharmaceutical agent that is given to the animal that contains the transfected cells. An example is a herpesviral TK gene, rendering cells that harbour it sensitive to killing by (for example) ganciclovir. Thus, cells treated with an agent to downregulate osteonectin can be used, according to the invention, as vaccine for tumour immunotherapy, e.g. especially for melanomas and metastatic tumours derived from melanomas, tumours of neural tissue, and/or mammary tumours and tumours of endothelial tissue; and e.g. for malignant tumours; ovarian cancers being less-preferred subjects of treatment. Thus the source cells before treatment to downregulate osteonectin can be of a wide range of types, including in particular the tissue types mentioned in reference [8] (Porter et al), and malignant tissue also of mesenchymal origin. According to the invention, downregulation of osteonectin can also be applied to the inhibition of angiogenesis for purposes of tumour treatment and other purposes.

Furthermore, according to the invention cytotoxic T-cells can be activated and/or expanded, e.g. in vitro, e.g. for purposes of cancer immunotherapy, by the use of transduced presenting or target cells, where the virus used for transduction of the presenting or target cells is a vector as described herein carrying genetic material encoding a downregulator of osteonectin, and in addition, if desired, encoding an immunomodulatory protein. After such treated cells have been used to activate and expand T-lymphocytes, the lymphocytes can subsequently be reinfused into a subject to be treated.

One method of using the vectors, provided by this invention, is to prepare a cellular immunogen such as a vaccine from tumour cells derived from one or more individuals by treating such tumour cells with a vector for downregulating osteonectin, and to administer this as an immunogen or vaccine for treatment of other subjects, e.g. patients. If a CTL response against the tumour cells is desired, however, for the reasons outlined above, the target antigens should be presented in the context of the correct MHC molecules. An immunogen or vaccine prepared from a tumour of one individual may not always therefore be appropriate for another individual with a different MHC type. Since MHC molecules vary from individual to individual, it is generally necessary, in order to activate CTL responses against the target antigens, to present the relevant target antigen to the immune system in the correct MHC context. Thus for use as an immunogen such as a therapeutic vaccine, in general it is considered that the selected target antigen is best introduced into the treated subject's or patient's own cells in order to generate an appropriate CTL response.

It can therefore be especially useful to base the tumour immunogen or vaccine on a patient's own tumour cells, a procedure known as autologous vaccination. A further major advantage of this way of use is that it can take advantage of antigenic targets that may be unique to a particular tumour; it is considered that the deregulated cell cycle control that is the basis of tumour growth can, over a period of time, lead to the accumulation of genetic changes manifested as new antigenic determinants. In this connection, the last-mentioned embodiments of the present invention can avoid or solve a problem with autologous vaccination procedure, namely that autologous tumour cells are poorly immunogenic.

In particular embodiments given below by way of example the invention relates to the downregulation of osteonectin in malignant melanoma cells, for example human melanoma cells, and to the restraint of their tumorigenic capacity thereby.

The invention is further described by way of example without intent to limit its scope. Reference is made to the accompanying Figures as follows:

Figure 1. Decrease in SPARC and Gelatinase A (72 kDa) levels in IIB-MEL-LES clones transfected with SPARC antisense cDNA. The parts of Figure 1 indicate a) expression levels of 5 isolated clones including those selected for further studies; b) protein bands from media of L-CMV cells and the different clones, electrophoresis in a minichamber, detected using anti-SPARC IgG; c) comparative mRNA expression of SPARC and Gelatinase A expressed by the different clones; d) zymography of serum-free conditioned media from L-CMV cells and the different clones.

Figure 2. In vitro features of IIB-MEL-LES clones transfected with SPARC antisense cDNA: a) cell growth, cell counts, in vitro invasiveness (b), adhesiveness (c), and migration (d).

Figure 3. Gelatinase A expression in human melanoma cell lines. Gelatinolytic activity (a) and western analysis (b) of serum-free conditioned media from different cell lines; c) gelatinolytic activity of IIB-MEL-LES-serum free-conditioned media under various conditions.

Figure 4. Analysis and quantification of neutrophil infiltrate at injection sites of tumor cells in nude mice.

Examples of the invention and materials for use in the invention can be made inter alia with the help of polynucleotides (known per se) encoding osteonectin.

Plasmids encoding antisense oligodeoxyribonucleotide to human osteonectin:

cDNAs encoding human SPARC (osteonectin), and their cloning, sequence and DNA manipulation have been described for example by:- Mason IJ, Taylor A, Williams JG, Sage H, and Hogan BLM, EMBO J 1986; 5: 1465-1472 'Evidence from molecular cloning that SPARC, a major product of mouse parietal endoderm, is related to an endothelial culture shock glycoprotein of Mr 43,000' -- giving sequence data, and incorporated herein by reference; and Swaroop A, Hogan BLM, and Francke U; Genomics 1988; 2: 37-47: 'Molecular Analysis of the cDNA for human SPARC/ osteonectin/ BM-40: sequence, expression and localisation of the gene to chromosome 5q31-q33', also giving sequence data and incorporated herein by reference.

OL Podhajcer et al have described, in 'Comparative expression of the SPARC and stromelysin-3 genes in mammary tumors', The Breast 5 (1996) pp 13-20 [9], the preparation and cloning of a 1.6 kb DNA sequence encoding full-length human SPARC (osteonectin), and particularly for example its isolation and cloning in known expression vector pET-3b. The cDNA was isolated from a breast cancer cDNA library, and has a coding sequence corresponding to the coding sequence of the cDNA given in Figure 3 of A Swaroop et al (1988), cited below, and incorporated herein by reference. SPARC (osteonectin) antisense expression can be obtained from a 1.6 kb cDNA for SPARC extending for example from nucleotide 15 to nucleotide 1698 of the sequence given by Swaroop et al (op.cit.), or otherwise as described in reference [9], by excising a clone in the antisense orientation using HindIII and Apal, and subcloning this product in the same orientation in the HindIII-Apal sites of the commercially available mammalian expression vector Rc/CMV, driven by the cytomegalovirus promoter (Invitrogen Corp), to create a plasmid designated herein as pCMV/SP-AS.

Reagents useful for carrying out related manipulations are also described in the given references. Osteonectin/SPARC can be expressed as polypeptide when desired e.g. in a bacterial system, in order to raise rabbit antisera against SPARC/osteonectin, as described in reference [9].

An antisense oligodeoxyribonucleotide to SPARC/osteonectin can be prepared from the 1.6kb cDNA encoding SPARC/osteonectin by harvesting the pCMV/SP-AS plasmids mentioned above and digesting with HindIII and Apal, and can be purified using agarose gel.

Melanoma cells transfected with antisense oligodeoxyribonucleotide to human osteonectin: Melanoma cells can be transfected with plasmid pCMV/SP-AS as described above by coprecipitation of the DNA with calcium phosphate. Stable transfectants can be selected with geneticin (GIBCO-BRL, 0.5 mg/ml). Isolated clones can be obtained by ring cloning and

limiting dilution.

Genetically disabled herpesviral vector encoding antisense
oligodeoxyribonucleotide to human osteonectin:

5 Antisense DNA to human osteonectin can also be used together with techniques as described in specification WO 96/26267 (Cantab Pharmaceuticals Research Limited: Inglis et al) and adaptable readily to the construction of a genetically disabled herpesviral vector (a gH-deleted defective HSV2 virus vector) encoding antisense oligodeoxyribonucleotide to human osteonectin, as follows:

10 Antisense oligodeoxyribonucleotide to SPARC (osteonectin) (prepared as described above, by digestion of cloned plasmid pCMV/SP-AS with HindIII and Apal) can be prepared for cloning by (for example) engineering the DNA ends so that the polynucleotide to be inserted, provided with a suitable promoter, and preferably also a polyA signal to facilitate transport
15 of mRNA into the cytoplasm, in per-se known manner, has complementary ends to match (at the 5' end) a HindIII site and (at the 3' end) a EcoRI site.

Manipulations to effect these alterations in the SPARC antisense oligodeoxyribonucleotide can be carried out in per-se known manner based on standard procedures described in
20 'Molecular Cloning: A Laboratory Manual' eds. J Sambrook, E F Fritsch and T Maniatis, 2nd ed 1989, Cold Spring Harbor Laboratory Press.

The polynucleotide can then be inserted into a gH-deleted defective HSV2 viral vector by modifying the example description given in specification WO 96/26267. For example, the
25 prepared DNA (oligodeoxyribonucleotide) can be ligated into a cloning vector pcDNA3 (available from Invitrogen Corporation) between the HindIII and EcoRI sites, and cloned.

The resulting vector can be designated pcDNA3-hSPARC. This can be digested with EcoRI, and then with HindIII, blunt-ended, and cloned into vector pIMC14 as described in
30 specification WO 96/26267 (in place of the murine gene as used in connection with the description given on pages 28-29 of WO 96/26267).

The resulting cloning vector encoding antisense oligodeoxyribonucleotide to osteonectin can then be used in adaptations of other procedures described in specification WO 96/26267,
35 including making a gH-deleted defective HSV2 virus vector encoding antisense oligodeoxyribonucleotide to human osteonectin at the site of deletion of the gH gene.

Other techniques for use of antisense polynucleotides, known per se, are readily adaptable

to the specificity needed for the present application by using suitable nucleotide sequences, e.g. of at least about 12 nucleotides complementary in sequence to the sequence of the chosen osteonectin target; by choosing from among known promoters suitable to the cellular environment in which they are to be effective, and other measures known per se. For example, the sequence at the 5' end of the coding sequence of the cDNA given in the Swaroop et al (1988) paper cited above can be used alternatively or in addition to derive antisense sequences for blocking purposes as described herein.

Reference is made to further techniques for use of antisense RNA to disrupt expression of a target gene, as for example indicated (in connection with a sialidase gene) in specification WO 94/26908 (Genentech: TG Warner et al). Techniques for using antisense oligonucleotides capable of binding specifically to mRNA molecules are also indicated in specification WO 94/29342 (La Jolla Cancer Research Foundation and the Regents of the University of Michigan: R Sawada et al) (in particular connection with mRNA encoding human lamp-derived polypeptides). Techniques for antisense oligonucleotides complementary to target RNA are indicated in specification WO 94/29444 (Department of Health and Human Services: B Ensoli and R Gallo) (as applied to basic fibroblast growth factor RNA). Techniques for using antisense oligonucleotides having a sequence substantially complementary to an mRNA which is in turn complementary to a target nucleic acid, in order to inhibit the function or expression of the target, are indicated in WO 94/24864 (General Hospital Corporation: HE Blum et al), (as applied to inhibition of hepatitis B viral replication). A review of antisense techniques is given by D Mercola and JS Cohen, ch.7 pp 77-89 in in RE Sobol and KJ Scanlon (eds.) 'Internet Book of Gene Therapy: Cancer Therapeutics' (Appleton & Lange, Stamford, Connecticut, 1995). Applications to other target specificities are accessible by adaptation.

Techniques for using ribozymes to disrupt gene expression are also known per se. For example, techniques for making and administering ribozymes (or antisense oligonucleotides) in order to cleave a target mRNA or otherwise disrupt the expression of a target gene are indicated in specification WO 94/13793 (Apollon: CJ Pachuk et al) (as applied to ribozymes that target certain mRNAs relevant to leukemias). A review of ribozyme techniques is given in M Kashani-Sabet and KJ Scanlon, ch. 8 pp 91-101 in RE Sobol and KJ Scanlon (eds.) 'Internet Book of Gene Therapy: Cancer Therapeutics' (Appleton & Lange, Stamford, Connecticut, 1995). Here also, applications to other target specificities are readily accessible by adaptation. According to examples of the invention, antisense oligonucleotide technique can be used together with ribozyme technique to disrupt the expression of osteonectin in a target cell, and the corresponding nucleic acid can be delivered in any appropriate viral or non-viral vector, or as naked DNA. If desired, expression of more than

one gene can simultaneously be blocked by including suitable antisense sequences.

Characteristics of human melanoma cell lines used for study and demonstration:

5 Studies were carried out to compare the in vitro invasive capacity of different human melanoma cell lines [12]. In three different experiments, IIB-MEL-LES cells exhibited 5 to 10-fold higher capacity to traverse matrigel, compared with the other cell lines (IIB-MEL-LES: 50.5 \pm 8.4, IIB-MEL-IAN: 10.3 \pm 1.1 and IIB-MEL-J: 4.8 \pm 1.1 cells/field: mean \pm SD).

10 In another series of experiments, nude mice were xenografted with 2×10^6 - 10×10^6 cells corresponding to each cell line and tumour growth was followed. Starting from 3×10^6 cells, tumour growth was observed in 100% of mice injected with every melanoma cell line. Yet, differences in tumour growth rate were observed among the three cell lines. When 3×10^6 cells were injected, a mean tumour volume of 0.1 cm³ was observed after 34 \pm 3.3 days (mean \pm SD), 42.5 \pm 6.5 days and 51.5 \pm 5.7 days in mice injected with IIB-MEL-LES, IIB-MEL-IAN and IIB-MEL-J cells, respectively. Similarly, a mean tumour volume of 1 cm³ was reached after 46.6 \pm 4.2 days, 68.4 \pm 6.4 days and > 80 days in mice injected with IIB-MEL-LES, IIB-MEL-IAN and IIB-MEL-J cells, respectively.

20 Downregulation of SPARC expression:

Twelve clones derived from the most aggressive IIB-MEL-LES cell line and 16 from the less aggressive IIB-MEL-IAN cell line were analyzed for SPARC mRNA expression following SPARC antisense c-DNA transfection. Three IIB-MEL-LES-derived clones were selected (termed L-1G, L-1E and L1D) exhibiting 27%, 52% and 87% decrease in SPARC mRNA expression compared to L-CMV cells transfected with the vector alone (Fig. 1a). Similarly, 4 IIB-MEL-IAN-derived clones (termed I-1H, I-1N, I-1V) exhibiting 73%, 82%, 66% and 61% decrease in SPARC mRNA expression compared to I-CMV control cells were also selected (not shown).

30 SPARC produced by different normal cells migrates as a single band with an apparent Mr of 40-43 kDa [5,6]. In contrast, IIB-MEL-LES and IIB-MEL-IAN cells, as well as fresh melanoma samples, produced up to three additional protein species as a result of post-translational cleavage by a leupeptin-inhibitable protease (M.F.L. et al., submitted, and Fig. 1b). Western analysis revealed the complete disappearance of the low molecular weight bands in the three IIB-MEL-LES derived clones, and a gradual decrease in the major band expression levels (Fig. 1b). Parental and L-CMV cells secreted 0.13-0.2 μ g/ml/ 10^6 cells of SPARC. Clones L-1G, L-1E and L-1D secreted 0.07-0.08, 0.05-0.03 and 0.03-0.01 μ g/ml/ 10^6 of SPARC, corresponding to an average decrease in SPARC secretion of 54%.

76.5% and 88.5% respectively.

In vitro growth and invasive capacity of transfectants: Figure 2a shows the in vitro growth properties of the different IIB-MEL-LES transfectants. When plated at low cell density (1×10^4 cells per well of a 24-well plate) a slight delay in the onset of logarithmic growth of SPARC antisense-transfected clones was observed, compared to L-CMV cells. Once the different cell types reached the logarithmic phase no difference in their doubling time was observed. Cells plated at higher density (2×10^4 and 4×10^4 cells per well of a 24-well plate) showed no difference compared to L-CMV cells (not shown).

When the transfectants were further analyzed for their capacity to invade matrigel-coated membranes we found that clones L-1G and L-1E exhibited in average almost 70-80% reduction in their invasive ability, while clone L-1D was essentially non-invasive (Fig. 2b). Similarly clones I-1H, I-1N, I-1T and I-1V exhibited 55%, 88%, 88% and 81% reduction respectively in their in vitro invasive capacity compared to I-CMV cells.

Gelatinase A and the in vitro invasiveness of melanoma cells: Initial zymographic analysis of serum-free conditioned media obtained from the different cell lines demonstrated that the higher in vitro invasive capacity of IIB-MEL-LES cells correlated with gelatinase A (MMP-2) activity and protein levels (Fig. 3a and 3b). In addition, an antisera anti-gelatinase A which completely blocked its gelatinolytic activity (Fig. 3c) inhibited almost 50% IIB-MEL-LES cells invasive capacity. In view of the correlation between gelatinase A levels and the in vitro invasive capacity of IIB-MEL-LES cells, we studied gelatinase A levels in the different IIB-MEL-LES derived clones. Figures 1a and 1c show a decrease in the expression levels of gelatinase A mRNA following the down-regulation of SPARC mRNA levels in the different IIB-MEL-LES-derived clones, which was accompanied by a decreased gelatinase A activity (Fig. 1d).

Cell adhesion to Matrigel and migration: Tumour cell attachment to surrounding matrix is the initial step leading to pericellular matrix degradation. Similarly, tumour cell attachment to matrigel must precede invasion. We found that the 3 IIB-MEL-LES derived clones displayed a reduced capacity to adhere to matrigel, suggesting that the decrease in their in vitro invasive capacity may be associated, at least in part, with a diminished adhesion capacity (Fig. 2c). Interestingly, all the 4 IIB-MEL-LES-derived clones also showed a strong reduction in their ability to adhere to matrigel and 3 of them essentially did not adhere (I-1H: 50%, I-1T: 0%, I-1V 0% and I-1N 8% compared to I-CMV control cells). In addition, we found a slight but statistically significant inhibition in the in vitro chemokinetic migration of the three IIB-MEL-LES-derived clones compared to L-CMV cells (Fig. 2d).

In vivo tumorigenicity: The in vitro data prompted us to study whether decreased SPARC expression levels might affect melanoma cells tumorigenicity. Table 1 demonstrates tumour formation in all mice xenografted with L-CMV cells in two independent experiments. All mice injected with cells obtained from the 3 IIB-MEL-LES derived clones showed no sign of tumour formation, even after eight months (Table 1). Histologic analysis of L-1D injection site 24 hr after injection showed a statistically significant increase in the recruitment of segmented neutrophils compared to L-CMV injection site (Fig. 4a), and a massive tumour cell necrosis (not shown). After 72 hr, the L-1D injection site was still infiltrated by a significant number of segmented neutrophils (Fig. 4a and 4b) while only few neutrophils were visible in the L-CMV injection site (Fig. 4a and 4c). After 7 days, the LES-1D injection site showed no viable tumour cells (not shown). A similar increased recruitment of segmented neutrophils was observed at the site of injection of I-1T and I-1N cells compared with I-CMV cells. In the case of I-1N cells we found no viable tumour cells at the injection site after 72 hr, while the absence of viable cells at the injection site of I-1T cells was evident after 7 days (not shown).

Further methods useful in the performance of the invention are indicated as follows:

Cell Lines: The IIB-MEL-J, IIB-MEL-LES and IIB-MEL-IAN cells lines were established from metastatic melanomas and were routinely grown in melanoma medium without transferrin and epidermal growth factor [12]. HFL-1 human fibroblasts (ATCC, CCL153) were grown in DMEM, 10% FBS and antibiotics. Cultures were maintained at 37 degC in a 5% CO₂ humidified incubator.

SPARC antisense expression: A 1.6kb full length cDNA encoding for human SPARC extending from nucleotides 15-1698 was isolated and clones as described [9]. A clone in the antisense orientation was excised with Hind III and Apa I and subcloned in the same orientation in the Hind III-Apa I sites of the mammalian expression vector Rc/CMV driven by the cytomegalovirus promoter (Invitrogen), to create plasmid pCMV/SP-AS. Melanoma cells were transfected with pCMV/SP-AS by coprecipitation of the DNA with calcium phosphate. Stable transfectants were selected with geneticin (GIBCO-BRL, 0.5 mg/ml). Isolated clones were obtained by ring cloning and limiting dilution.

Preparation of SPARC antiserum, western and ELISA studies: A full length cDNA for human SPARC was subcloned into the expression vector pET-3b as described [9]. BL-21 cells transformed with pET-3b plasmid or with pET-3b/SPARC plasmid were grown and induced by 1mM IPTG. The bacterial soluble fraction containing recombinant SPARC was concentrated by adding ammonium sulphate to 80% saturation. After centrifugation, the

pellet was resuspended in lysis buffer containing 6M urea, loaded on a DEAE Sephacel column (Pharmacia, Sweden) followed by protein elution with a discontinuous NaCl gradient. SPARC eluted at 200 mM NaCl concentration was dialyzed against PBS containing gradually decreasing concentrations of urea until it was completely removed.

5 This sample was used to raise a rabbit antiserum by standard procedures [36]. The Protein G Sepharose-affinity purified-IgG fraction (Pharmacia, Sweden) recognized one band of 42 kDa in serum-free conditioned medium of HFL-1 cells corresponding to the expected Mr of SPARC and did not react with serum-free conditioned media of the human breast cancer cell lines MCF-7 and MDA-MB-231 which did not express SPARC mRNA (M.F.L. et al.,
10 submitted).

Nearly confluent human melanoma cells were washed twice with PBS and kept in serum-free medium. After 24 hr the medium was collected, centrifuged for 10 min at 1,500 rpm to remove debris and concentrated with ammonium sulfate. The precipitate was dissolved
15 in PBS in the presence of protease inhibitors (PMSF and EDTA, 1 mM final concentration) and dialyzed against the same buffer. Samples were separated by reduced SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with skimmed milk in PBS, membranes were incubated overnight at 4 degC with affinity purified antiserum raised against human rSPARC (at 1/1000 dilution) or rabbit antiserum anti-human gelatinases.
20 After washing with 0.05% Tween-20 in PBS, the membranes were further incubated for 2 hr at room temperature with alkaline phosphatase-labelled goat anti-rabbit IgG (Jackson Immuno Research). Bands were detected using NBT-BCIP following manufacturer's instructions (GIBCO-BRL).

25 For ELISA studies, 30ul per well of concentrated conditioned media was seeded in duplicate in 96-well-polyvinyl ELISA plates and dried overnight at 37 degC. Non specific binding was blocked with 5% skimmed milk over 2 hr at room temperature and samples were incubated overnight at 4 degC with anti-SPARC antiserum (at 1/250 dilution). After washing with 0.1% BSA-BPS, SPARC was detected using a peroxidase-conjugated swine Ig anti-rabbit
30 1g (Dako). After washing, the reaction was developed with o-phenylenediamine and the absorbance read at 415 nm. The results were obtained from a standard curve with purified rSPARC (range 0-50 ug/ml). Three experiments using different batches of conditioned media were performed.

35 Zymography: Analysis of gelatinolytic activity was performed using 10% SDS-PAGE gels containing 0.1% gelatin. One part of serum-free conditioned media obtained as described was mixed with 1 part of Laemmli's buffer (without beta-mercaptoethanol) and incubated for 30 min at room temperature before loading in the gel. At the end of the electrophoresis,

the gels were washed 30 min each at room temperature, with the following solutions: a) 50mM Tris-HCl, pH 7.5, 2.5 % Triton X-100; b) 50mM Tris-HCl, pH 7.5, 2.5% Triton X-100, 5 mM CaCl₂, 1 μ M ZnCl₂. c) 50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 1 μ M ZnCl₂, followed by an overnight incubation at 37 degC in 50mM Tris-HCl, pH 7.5, 5mM CaCl₂, 1 μ M ZnCl₂. After staining with Coomassie Brilliant Blue R-250, gels were destained and photographed.

When the effect of the antisera against gelatinases was tested, conditioned media was preincubated during 45 min at room temperature with the antiserum before loading in the gel [37].

Invasion, adhesion and migration assays: The invasiveness of parental and transfected cells was determined in 24-transwell chambers (Costar). PVP-free polycarbonate filters, 8- μ m pore size, were coated with 5 μ g of Matrigel and dried under a hood. After adding 600 μ l of medium (DMEM:0.1% BSA) in a cluster plate well, coated transwells were mounted in the chamber. Cells were collected with a short exposure to citrate buffer (0.13 M sodium citrate and 0.015 M KCl), washed with PBS, resuspended at 1×10^6 cells/ml in DMEM:0.1% BSA buffer, and 0.1 ml were added to the inside of the transwell. After 16-18 hr incubation at 37 degC in 5% CO₂, the cells on the upper face of the filter were removed with a cotton swab. Cells attached to the lower face of the filters were fixed in methanol, stained with hematoxylin and counted. To test the involvement of gelatinases, invasion assays were performed in the presence of different dilutions of anti-gelatinase antisera. The migration assays were performed in a similar way without materiel coating, after soaking polycarbonate filters in gelatin to increase cell adhesion to the filters [38]. Serum free-conditioned media obtained from HFL-1 fibroblasts was used as chemoattractant.

For adhesion assays, the plastic surface of 96-well microtiter plates was coated with 5 μ g of matrigel and dried under a hood. Cells collected with citrate buffer were washed with PBS, resuspended at 2.5×10^5 cells/ml in DMEM and 100 μ l added to each well. At different times well plates were washed with PBS to removed unattached cells and fixed with paraformaldehyde. Ten random fields/wells were counted under phase contrast microscopy. Each experiment was performed at least in triplicate.

Table 1 shows in-vivo tumorigenic capacity of IIB-MEL-LES cell clones with stable expression of SPARC antisense RNA. 8-12 week old, male athymic balb/c nu/nu mice received into the left flank s.c. injections of 3×10^6 cells, in a total volume of 0.1 ml. Tumor volume was measured in the two perpendicular diameters with calipers. Mice were obtained from the National Commission of Atomic Energy, Buenos Aires. Experiments 1

and 2 were terminated after 240 and 210 days respectively. The data are expressed as the number of mice which developed tumors of 1.5 cm^3 , of total mice.

	Control		Cell Clones	
	L-CMV	L-1G	L-1E	L-1D
Experiment 1	6/6	0/6	0/6	0/6
Experiment 2	7/7	0/7	0/7	0/7

Further results are shown in accompanying Figures 1-4:-

Figure 1 indicates decrease in SPARC and Gelatinase A (72 kDa) levels in IIB-MEL-LES clones transfected with SPARC antisense cDNA. a) 10ug of total RNA were electrophoresed in 1% agarose gels in the presence of formaldehyde, blotted into nylon membranes and hybridized with probes corresponding to SPARC and 36B4 [9]. The nylon membrane was washed and re-hybridized with a probe corresponding to gelatinase A probe extending from nucleotides 142 to 2150 [39]. Autoradiography was for 24 hr for SPARC and 36B4 and 96 hr for gelatinase A. Only the expression levels of 5 isolated clones are shown, including those selected for further studies. b) 15 ug of total protein obtained from serum-free conditioned media of L-CMV cells and the different clones was loaded in each lane and electrophoresed in a minichamber. Protein bands were detected using affinity purified anti-SPARC IgG. Molecular weight markers are indicated in the left. c) Comparative mRNA expression of SPARC and Gelatinase A expressed by the different clones after quantification by two-dimensional densitometric scanning with an ULTROSAN XL densitometer (Pharmacia, Sweden) and normalized by dividing by the 36B4 signal for each sample. d) Zymography at neutral pH of serum-free conditioned media obtained from L-CMV cells and the different clones. The migration of the 72 kDa and the 92 kDa gelatinases is shown.

Figure 2 shows in-vitro features of IIB-MEL-LES clones transfected with SPARC antisense cDNA. a) cells were trypsinized, washed and 1×10^4 cells plated in each well of 24-well plates in triplicates in medium without selecting drug. Cell growth was followed until cells detached from the plastic. Cell counts were performed with a hemocytometer. Cells corresponding to each of the selected clones and L-CMV cells were studied for in vitro invasiveness (b), adhesiveness (c), and migration (d). The experimental details are described in methods section. The significance of differences was determined using the One Way Analysis of Variance (ANOVA) and Tukey-Kramer Multiple Comparison Tests, $p < 0.001$ (*); $p < 0.01$ (**); $p < 0.05$ (***).

Figure 3 shows gelatinase A expression in human melanoma cell lines. Gelatinolytic activity (a) and western analysis (b) of serum-free conditioned media obtained from different cell lines; c) gelatinolytic activity of IIB-MEL-LES-serum free-conditioned media in the presence of 72 kDa antiserum: 1) no serum added; 2) 1/50 dilution of 72 kDa antiserum; 3) 1/100 dilution of 72 kDa antiserum. See methods section for details.

Figure 4 shows analysis and quantification of the neutrophil infiltrate at the injection sites of tumor cells in nude mice. Cells were injected in the left flank of nude mice. The site of injection was removed after 24 hr and 72 hr, paraffin-embedded, cut and stained. a) 5-10 different fields corresponding to microphotographs (at 1000x magnification) were used for calculating the neutrophil infiltrate. Data represent the means \pm s.d (bars): $p < 0.001$. b) Microphotographs showing the site of injection after 72 hr of: b) L-ID cells; c) L-CMV cells; N: segmented neutrophils; T: tumor cells.

It has also been found, in accordance with the invention, that L-ID cells in which SPARC/osteonectin has been downregulated in accordance with the description herein, can abrogate tumour growth of parental cells, for example when xenografted into nude mice as mixed cells in doses of about 3×10^6 parental cells, using ratios of cell numbers of the respective types of up to e.g. 4:1 (parental cells:L-ID cells) and following tumour outcome for 4 months.

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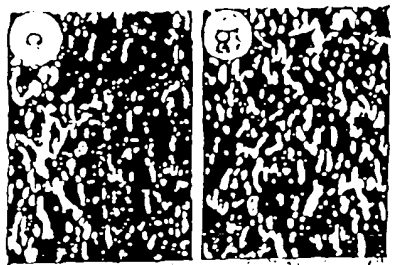
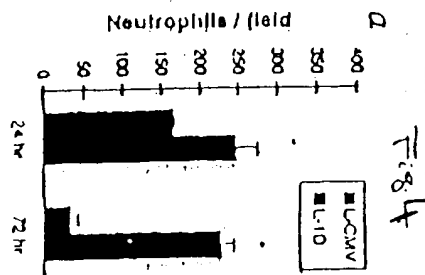
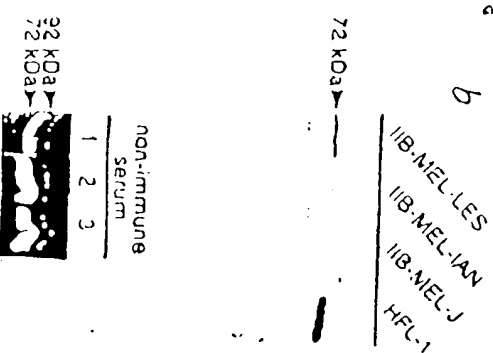
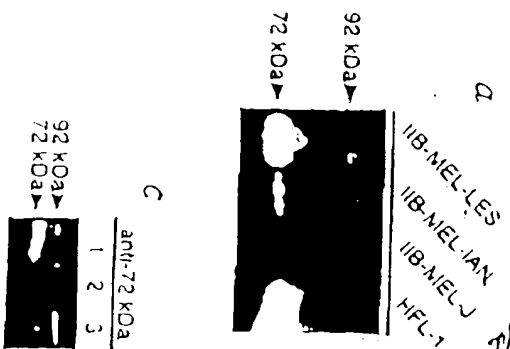
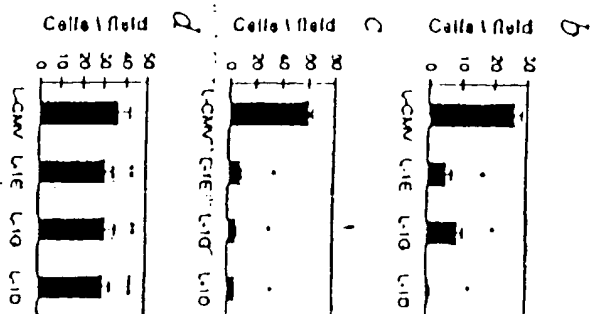
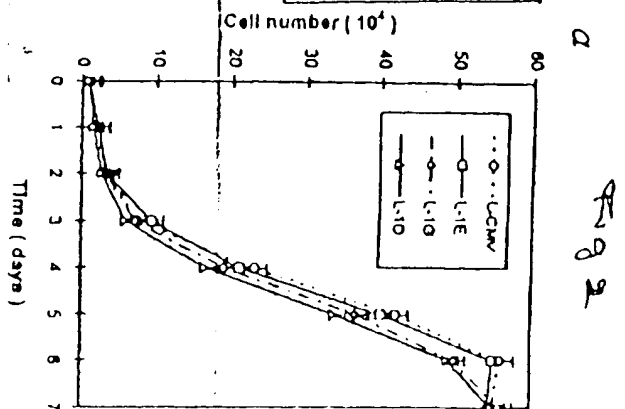
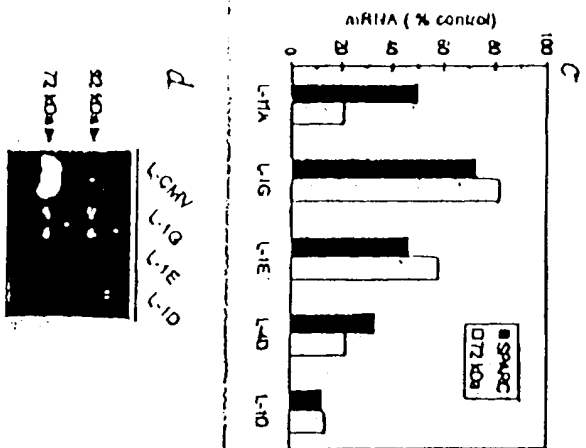
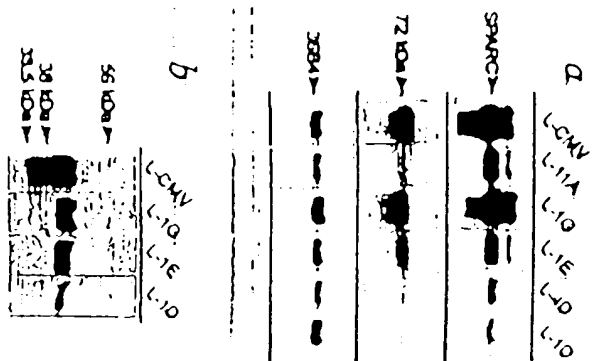
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15 The invention is susceptible of numerous modifications and variations which will be apparent to the person skilled in the art in the light of this disclosure. The scope of the present disclosure is intended to include modifications and variations, as well as combinations and subcombinations of the features mentioned herein. All documents cited are hereby incorporated by reference in their entirety.





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